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LPS-induced knee-joint reactive arthritis and spinal cord glial activation were reduced after intrathecal thalidomide injection in rats

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ABSTRACT

Aims: Thalidomide is thought to prevent TNF-α production, and such mechanism could be useful in a spinally delivered drug approach for the control of peripheral inflammation. This study aimed to evaluate the effect of intrathecal thalidomide, in comparison with that of intraperitoneal treatment, on articular incapacitation, edema, synovial leukocyte content, and spinal cord glial activation in a model of *Escherichia coli* lipopolysaccharide (LPS)-induced reactive arthritis in rats.
Main methods: LPS (30 ng) was injected into a knee-joint previously primed with carrageenan (300 μg). Systemic (30 and 100 mg/kg; intraperitoneal, i.p.) and intrathecal (10 and 100 μg; i.t.) thalidomide were given 1 h or 20 min before LPS injection, respectively. Articular incapacitation and edema were evaluated hourly. After 6 h, synovial fluid and lumbar spinal cords were collected for subsequent evaluations of cell migration and expression of CD11b/c and GFAP markers, respectively.
Key findings: Systemic (30 and 100 mg/kg) or intrathecal (10 and 100 μg) thalidomide reduced articular incapacitation, edema, and polymorphonuclear migration. In addition, i.p. and i.t. thalidomide reduced the expression of CD11b/c and GFAP markers in the lumbar spinal cord. These results suggest that thalidomide can also produce peripheral anti-inflammatory effects through action in the spinal cord that may involve glia inhibition.
Significance: This study provides new evidence that the direct spinal delivery of immunomodulators may be an alternative for the treatment of arthritic diseases, which require long systemic treatment with drugs associated with undesirable side effects.

Introduction

Thalidomide has been shown to be effective in arthritis treatment, and may constitute an alternative when the disease is unresponsive to non-steroidal anti-inflammatory drugs, or when the patient cannot tolerate corticosteroidal side effects (Wei et al. 2003). Patients exhibiting ankylosing spondylitis and juvenile rheumatoid arthritis symptomatology reported significant improvement after thalidomide therapy (Lehman et al. 2002; García-Carrasco et al. 2007). The benefits achieved through thalidomide administration in patients with arthritis include the relief of peripheral pain and inflammation (Huang et al. 2002; Goli 2007), and such effects have been demonstrated in different animal models as well (Ribeiro et al. 2000; Vale et al. 2006; Rocha et al. 2006). One of the main mechanisms underlying thalidomide's mode of action is the inhibition of tumor necrosis factor-alpha (TNF-α) production by amplifying its mRNA degradation (Sampaio et al. 1991; Moreira et al. 1993). TNF-α is an important cytokine involved in the modulation of peripheral pain and inflammation of the joints (Tonussi and Ferreira 1999; Bressan et al. 2006). In addition to its peripheral action, TNF-α released by activated glia in the lumbar spinal cord contributes to exaggerated pain (Milligan 2001) probably through a direct action on the central terminals of nociceptors (Parada et al. 2003). Since the central sensitization of primary afferents is thought to contribute to peripheral inflammation (Willis 1999), we hypothesized that in addition to a direct action on inflammatory cells thalidomide can also produce peripheral effects by acting at the spinal cord level. Therefore, the aim of this study was to evaluate the effect of intrathecal thalidomide, in comparison with that of intraperitoneal treatment, on articular incapacitation, edema, synovial leukocyte content, and spinal cord glial activation in a model of *Escherichia coli* lipopolysaccharide (LPS)-induced reactive arthritis in rats.

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Material and methods

Animals

This study was performed on 66 male Wistar rats (250–300 g), which were housed in temperature-controlled rooms (21 ± 2 °C) under a 12/12 h light/dark cycle with free access to water and food until the onset of experimental sessions. All experiments were conducted according to the ethical guidelines of the International Association for the Study of Pain (IASP 1983), and approved by the local committee for the ethical use of animals (CEUA-UFSC).

Drugs

Thalidomide was synthesized and kindly donated by Prof. Dr. Paulo Cesar Leal (FUNED, Brazil). Thalidomide was administered as a pretreatment 60-min (intraperitoneally) or 20-min (intrathecally or subcutaneously) before the LPS stimulus. The control group received only vehicle (1% dimethylsulfoxide in PBS, pH 7.4).

Carrageenan/LPS-induced reactive arthritis

The carrageenan/LPS-induced reactive arthritis model is described in detail elsewhere (Bressan et al. 2006). Briefly, 20 μl of a boiled, 15 mg/ml carrageenan solution (BDH Chemicals Ltd., UK), suspended in sterile saline 0.9%, was injected into the right knee-joints of rats (300 μg/20 μl/knee; i.art.). All rats received the carrageenan priming. The injection site was previously shaved and treated with 10% povidone-iodine solution. The second challenge was carried out in this joint with *E. coli* lipopolysaccharide (LPS; 30 ng) serotype 055:B5 (DIFCO, USA) diluted in physiological saline (0.9%), in a volume not exceeding 50 μl per injection, 3 days after the previous carrageenan sensitizing stimulus. Without the carrageenan priming, this amount of LPS (30 ng) did not induce incapacitation or edema. The intra-articular injections were administered without sedation. To avoid major distress the experimental animals were gently handled with the aid of a soft rubber cone, kept calm in a supine position, and intra-articular injections were quickly given with 26-gauge needles.

Algesimetric test

Arthritic nociception was evaluated by the rat knee-joint incapacitation test (Tonussi and Ferreira 1992). The rats were placed on a revolving cylinder (30 cm diameter; 3 rpm) for 1-min periods, while a computer-assisted device measured the total time the right hind paw was not in contact with the cylinder surface — the paw elevation time (PET, s). Before the LPS stimulus, the PET was 10 to 15 s. LPS increases PET only in the affected limb (Bressan et al. 2006). The paw elevation time was evaluated immediately before (time 0) and hourly after LPS injection for 6 consecutive hours (times 1 to 6).

Motor impairment assessment

When cylinder starts revolving, the animals promptly and spontaneously walk to keep themselves on the top, without assistance of the experimenter. Each experiment comprised 7 periods of gait registering for each animal. Any repeated delay to react to the cylinder movement, or the animal fall, were considered motor impairment. When a treatment was stated as causing no motor impairment was because the above mentioned events did not occur or their occurrence frequency was not different from naïve animals.

Edema measurement

In order to quantify the inflammatory edema induced by LPS, the rats were restrained as described above. A micrometer was used to measure the articular diameter of the inflamed knee-joint through the medio-lateral axis. The articular diameter was taken after each paw elevation time measurement. The data are presented as the difference between the articular diameter values taken hourly after LPS injection and values taken just before LPS injection in millimeters (mm).

Spinal cord immunohistochemistry

Six hours after the data recording (incapacitation and edema), the rats were deeply anesthetized with 15% chloral hydrate (0.5 g/kg, i.p., Vetec Química Fina LTDA, Rio de Janeiro, RJ, Brazil) and perfused transcardially with cold phosphate buffered saline (PBS; 0.1 M; pH 7.4; 300 ml) followed by cold buffered paraformaldehyde (PFA; 4%; pH 7.4; 500 ml, Vetec Química Fina LTDA, Rio de Janeiro, RJ, Brazil). The lumbar spinal cord segment (L4–L5) was removed after perfusion, post-fixed for 4 h in 4% PFA (4 °C), cryoprotected overnight in 30% sucrose/PBS (4 °C), frozen in O.C.T. embedding medium (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and stored at −80 °C until use. Thin (12 μm) cryosections ($n = 6$–8 sections/animal) from each treatment group were cut with a cryostat (CM 1850, Leica), thaw-mounted onto glass slides, and processed simultaneously. The sections were washed with PBS (3 × 5 min), and incubated at room temperature in PBS containing 0.1% Triton X-100 for 30 min. To avoid non-specific labeling, slides were incubated at room temperature for 1 h in PBS containing 5% normal bovine serum (NBS) and 1% bovine serum albumin. Subsequently, sections were incubated overnight at 4 °C with the primary antibodies: polyclonal rabbit anti-GFAP (1:500; Dako Cytomation, Carpinteria, USA) and monoclonal mouse anti-rat CD 11b/c (OX-42), which recognizes the complement receptor CR3 (1:200; Invitrogen, New York, USA). After incubation, the slides were washed with PBS (3 × 5 min) and incubated for 2 h at room temperature (23 ± 2 °C) with the secondary antibodies: goat anti-rabbit IgG FITC conjugate (1:100; Sigma, St. Louis, USA) and cross-adsorbed with goat anti-mouse IgG Alexa Fluor Red 546 (1:500; Invitrogen, New York, USA). The antibodies were diluted in PBS containing 2% NBS. Following the incubation, the slides were washed with PBS (3 × 5 min), cover-slips were mounted in buffered glycerin (pH 9) and visualized with a fluorescent light microscopy set-up. To minimize variability in staining, tissue from all treatment groups were run in the same immunohistochemical session. A negative control omitting the primary antibody was performed for all experiments.

Image capture and analysis

Images were captured with an epifluorescence light microscope (Olympus, BX-41) equipped with adequate filters for FITC and Alexa Fluor 546 (U-MWG2 and U-MWB2, Olympus), a digital camera (3.3 Mpixel QCOLOR3C, Qimaging™) and image acquisition software (Qcapture Pro 5.1, Qimaging™). The slides were visualized under a 10× magnification objective and gray scale, 12-bit images without over- and/or undersaturated pixels were captured in a predefined region of interest in the lumbar dorsal horn (L4–L5) including laminae I and II (superficial laminae). Anatomical landmarks (Molander et al. 1984) were used to capture the same region within the spinal cord dorsal horn in different tissue sections. A mean reading of 6–8 spinal cord sections from 6 animals representative of each of the test conditions (naive, carrageenan/LPS and carrageenan/LPS/thalidomide treated rats) were analyzed and the quantitative methods were assessed in a blinded fashion. Identical camera and microscope acquisition settings were used throughout the experiment.

Background noise images were captured without UV lamp excitation and were later subtracted from images showing fluorescent immunoreactivity. Additionally, reference images were taken from standard, homogeneously fluorescent slides (Chroma, Rockingham, USA) against which the immunoreactive signal was normalized, thereby making the tissue sample images acquired in different